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Plant ascorbate peroxidase: molecular phylogeny and role in oxidative stress

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SUMMARY. Oxidative stress appears as a condition in accumulation and detoxification of reactive oxygen species (ROS). ROS are oxygen-derived free radicals, generated predominantly in mitochondria, peroxisomes and chloroplasts, as natural byproducts of the normal cell aerobic metabolism. In spite of their damaging effect, ROS can act as secondary messengers in different cellular processes, including tolerance to environmental stress factors. To neutralize the harmful effects of ROS, plants have evolved enzymatic and non-enzymatic defense systems. In flowering plants, ascorbate peroxidase (APX) is present in eight isoenzyme forms and constitutes an important enzymatic component in scavenging the harmful hydrogen peroxide to water as part of ascorbate-glutathione cycle. APX proteins, their roles, *in planta* expression location and their phylogenetic relationships are presented in the current paper. The phylogenetic analysis performed with the maximum likelihood method which was established for 118 protein sequences of 45 flowering plants. Our phylogenetic analysis revealed diversification of ascorbate peroxidase in angiosperms, and indicates a close relationship of APX1 with APX2, APX3 with APX4 and APX5, and APX6 with sAPX and tAPX proteins. Evolutionary relationships of plant ascorbate peroxidase isoenzymes indicate the evolution of different plant species genome and their phylogenetic affiliation.

Keywords: antioxidative defense system, evolution, osmoprotectants, phylogenetic

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Introduction

Over the course of their lifetime, plants are exposed to different adverse environmental conditions, like osmotic and oxidative stress. In order to protect themselves against harsh external conditions, plants accumulate a series of protecting compounds, called osmoprotectants, and activate their antioxidative defense system. Appearance of O_2-evolving photosynthetic organisms and aerobic metabolism inevitably generated the occurrence of highly reactive oxygen species (ROS) (Halliwell 2006). During photosynthesis, oxygen is generated in the chloroplasts, and can accept electrons, thus forming $O_2^{\cdot -}$ (superoxide radical). In a multistep reaction, different types of ROS are generated from ground state oxygen i.e. $O_2^{\cdot -}$ (superoxide radical) and leads to the formation of H_2O_2 (hydrogen peroxide), $^1O_2$ (singlet oxygen), $HO_2^{\cdot}$ (perhydroxy radical), $HO^{\cdot}$ (hydroxyl radical), ROOH (alkyl hydroperoxide radical), $ROO^{\cdot}$ (alkylperoxyl radical) and $RO^{\cdot}$ (alkoxyl radical), which are highly reactive molecules causing serious damage to cell components and DNA, conducting to cell death (Gill and Tuteja, 2010). Under steady state conditions, damaging ROS molecules are scavenged by a set of antioxidative defense systems characteristic to chloroplasts, mitochondria and peroxisomes (Foyer and Harbinson, 1994; Alscher *et al.,* 1997; Klotz, 2002; Apel and Hirt, 2004; Navrot *et al.,* 2007; Heyno *et al.,* 2011; Sharma *et al.,* 2012), thus maintaining equilibrium between production and scavenging of ROS. This equilibrium can be disturbed by various biotic and abiotic environmental stress factors, such as pathogen attacks, salinity, drought, extreme temperatures, intense light, heavy metals, air pollution, herbicides and mechanical stress. Due to adverse stress factors, the levels of ROS in cells can suddenly increase and cause serious cell structure damages (Elstner, 1991; Malan *et al.,* 1990; Tsugane *et al.,* 1999).

Plant antioxidative defense mechanisms are of two types: enzymatic and non-enzymatic. Enzymatic system includes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and enzymes of ascorbate-glutathione (AsA-GSH) cycle, such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Non-enzymatic system comprises of ascorbate (AsA), glutathione (GSH), tocopherols, carotenoids, and flavonoids (Noctor and Foyer, 1998; Sharma *et al.,* 2012) (Fig. 1).

It is important to mention that the osmoprotectant molecule, proline, can act as a non-enzymatic antioxidant needed to counteract the damaging effects of ROS in organisms as microbes, plants and animals (Chen and Dickman, 2005; Székely *et al.,* 2008). Despite their deleterious effect, ROS can act as secondary messengers in different cellular processes, including tolerance to environmental stresses (Desikan *et al.,* 2001; Yan *et al.,* 2007; Sharma *et al.,* 2012). The aim of this article is to present the plant ascorbate peroxidases (APX) (EC 1.11.1.11) phylogeny, as a main participant in neutralizing ROS.

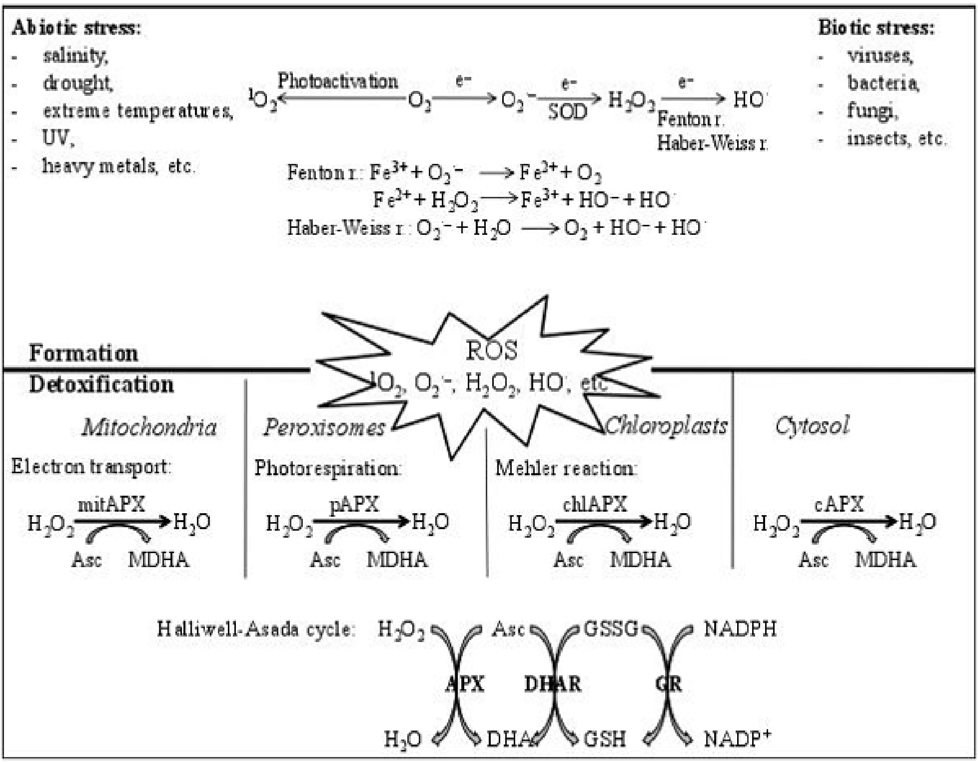

Figure 1. Schematic representation of APX role in ROS formation and detoxification in plants; ROS formation: $^1O_2$: singlet oxygen, O_2: molecular oxygen, $O_2^{\cdot-}$: superoxide radical, SOD: superoxide dismutase, H_2O_2: hydrogen peroxide, $HO^{\cdot}$: hydroxyl radical. ROS detoxification: Asc: ascorbate, MDHA: monodehydroascorbate; Halliwell-Asada cycle: H_2O_2: hydrogen peroxide, APX: ascorbate peroxidase, H_2O: water, DHAR: dehydroascorbate reductase, DHA: dehydroascorbate, GSSG: oxidized glutathione, GR: glutathione reductase, GSH: reduced glutathione, NADPH: reduced nicotinamide adenine dinucleotide phosphate, $NADP^+$: nicotinamide adenine dinucleotide phosphate (based on Noctor and Foyer, 1998; Sharma *et al.,* 2012).

Role of APX enzyme in antioxidative defense system during stress conditions

In plants, APX is the most distributed antioxidant enzyme and is considered to be a key ROS scavenger enzyme and cell protecting molecule (Orvar and Ellis, 1997). APX, together with catalase, controls the level of H_2O_2 in cells, but the main H_2O_2 scavenging role is thought to belong to APX, which converts H_2O_2 to H_2O in water-water and AsA-GSH (ascorbate glutathione or Halliwell Asada) cycles. APX uses two molecules of AsA to reduce H_2O_2 to water with subsequent generation of two molecules of MDHA (monodehydroascorbate) (Fig. 1).

Based on amino acid sequences, several different isoforms of APX family have been found at different subcellular localization in flowering plants, including chloroplast, mitochondria, peroxisomes and cytosol (Jimenez *et al.,* 1997; Madhusudhan *et al.,* 2003; Sharma and Dubey, 2004; Nakano and Asada, 1987). The organelle APX is efficient in scavenging H_2O_2 produced in the organelles, while cytosolic APX neutralizes H_2O_2 from cytosol, apoplast and that diffused from organelles (Sharma *et al.,* 2012). APX isoforms have a much higher affinity for H_2O_2 compared to CAT and are essential in scavenging ROS during stress conditions (Wang *et al.,* 1999). Many publications reported enhanced level of APX enzyme activity during different abiotic stress conditions, such as salinity, drought, extreme temperatures, heavy metal toxicity and presence of high-light intensities (Boo and Jung, 1999; Sharma and Dubey, 2005a; Sharma and Dubey, 2007; Han *et al.,* 2009; Maheshwari and Dubey, 2009; Hefny and Abdel-Kader, 2009). Begara-Morales *et al.,* (2013) reported the increase of APX enzyme activity in pea plants grown under saline (150 mM NaCl) conditions, *Anabaena doliolum* also revealed enhanced APX activity during salt stress (Srivastava *et al.,* 2005), water stress induced APX activity in three cultivars of *Phaseolus vulgaris* (Zlatev *et al.,* 2006) and *P. asperata* (Yang *et al.,* 2008), mild drought stress in rice generated higher chloroplastic-APX activity (Sharma and Dubey, 2005b), Cd stress caused increased APX activity in leaves of *Ceratophyllum demersum* (Arvind and Prasad, 2003), *Brassica juncea* (Mobin and Chan, 2007), *Triticum aestivum* (Khan *et al.,* 2007) and *Vigna mungo* (Singh *et al.,* 2008). Simonovicova *et al.,* (2004) reported enhancement of APX activity in *Hordeum vulgare* roots in the presence of Al stress. However, biochemical methods currently used to assess the enzyme APX activity can evaluate only the total APX activity, without distinguishing between the activities of the different APX isoforms.

Materials and methods

Data collection

The ascorbate peroxidase protein sequences used in this paper were gathered from the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) (McEntyre and Ostell, 2002) database and from arabidopsis.org. The sequences were run through BLAST Sequence Analysis Tool online program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in a non-redundant database (McEntyre and Ostell, 2002).

Sequence similarity

APX sequence similarity between *Arabidopsis thaliana* and the studied species was calculated using matGAT software with first gap penalty 12 and expanding gap 2 settings. (Campanella *et al.,* 2003). We collected separately the monocots and dicots APX sequences, the sequences which did not correspond to homology were not taken into account. Thus, the similarity score was obtained from comparing 118 protein sequence. The scoring matrix used was BLOSUM50, penalties for first gap 16, and extending gap 4. The query monocots sequences were assessed comparing their similarity with *Arabidopsis thaliana* APXs (reference sequences: P82281, Q05431, Q1PER6, Q42564, Q42592, Q42593, Q7XZP5, Q8GY91). The predicted subcellular localization was obtained from http://cello.life.nctu.edu.tw/ (Yu *et al.,* 2004; Yu *et al.,* 2006) with the specific parameters ("Eukaryotes" and "Protein").

Magnoliophyta APX proteins

APX protein forms of 45 species from Magnoliophyta phylum were analyzed and compared to each other. The protein sequences were classified based on Catalogue of Life database (Table 2). Magnoliopsida class analysis indicated several families: Brassicaceae and Capparaceae family (APX1-3 and APX5); Capparaceae and Chenopodiaceae family (APX1-3 and APX6); Cucurbitaceae family (APX1,2 and APX6); Euphorbiaceae family (APX1, APX3, APX6); Fabaceae family (APX2) Malvaceae (APX1-2); Moraceae (APX1), Nelumbonaceae (APX2); Pedaliaceae and Rosaceae family (APX1-3, APX6), Rutaceae (APX1-3); Salicaceae (APX1-2, APX6); Solanaceae (APX1, APX3, APX6), Sterculiaceae family (APX2); Vitaceae family (APX1-2, APX6). From Liliopsida class Poaceae family (APX1-2, APX4, APX6, sAPX, tAPX) was analyzed.

Classification of APX isoforms

In order to fulfil an accurate analysis, a table containing the eight *Arabidopsis thaliana* AtAPX isoforms was created, as a benchmark for all APX proteins of the examined species (Table 1). The APX protein sequences were grouped according to their similarity index. Table 1 shows the APX isoforms according to NCBI database and the same isoforms based on their sequence alignments. The highest APX similarity values were used in our analyses. Similarity values below 50% were not considered.

Phylogenetic tree

For phylogenetic analyses 118 sequences were used: APX1 (25 sequences), APX2 (26 sequences), APX3 (10 sequences), APX4 (11 sequences), APX5 (11 sequences), APX6 (15 sequences), sAPX (10 sequences), tAPX (10 sequences) (Table 1). The protein sequences were sorted and aligned by multiple sequence alignments with ClustalW in MEGA7 program (Kumar *et al.,* 2016) using default settings. The length of aligned protein sequences were 271 amino acids. The phylogenetic tree was generated in PhyML SMS (Guindon *et al.,* 2010; Lefort *et al.,* 2017) maximum likelihood framework program. The phylogenetic reconstruction was performed assuming a LG +G+I (Le and Gascuel, 2008) evolution model with gamma distributed variation rate across site (G) and a proportion of invariable site (I). The statistics value was based on Shimodaira–Hasegawa [SH] approximate likelihood ratio test [aLRT]. Phylogenetic tree was created by FigTree (v 1.4.0.) program. Main genetic distance between groups and within groups was estimated using p-distance (bootstrap value 1000) by MEGA7 program.

Results and discussion

Table 1 summarizes the APX isoforms, their cellular localization, role and expression location in *A. thaliana*.

Table 1.

APX isoforms and their role in *Arabidopsis thaliana* according to arabidopsis.org

APX protein	TAIR accession no.	Cellular localization	Role induced by	*In-planta* expression location
APX1	At1g07890	Golgi apparatus, chloroplast stroma, cytoplasm, plasma membrane, plasmodesma, cell wall	Cd ion, cytokinin, heat, oxidative stress, reactive oxygen species, salt stress	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen, pollen tube cell,) fruit, seed, guard cell
APX2	At3g09640	cytoplasm	hydrogen peroxide catabolic process, oxidation-reduction process, oxidative stress	leaf
APX3	At4g35000	chloroplast envelope, glyoxysomal membrane, mitochondrion, peroxisomal membrane, plasmodesma, plastid, vacuolar membrane	hydrogen peroxide catabolic process, oxidation-reduction process, cytokinin, oxidative stress	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen, pollen tube cell,) seed, guard cell

APX protein	TAIR accession no.	Cellular localization	Role induced by	*In-planta* expression location
APX4	At4g09010	chloroplast thylakoid lumen, chloroplast thylakoid membrane, cytoplasm, nucleus	oxidation-reduction process, oxidative stress	shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell
APX5	At4g35970	integral component of peroxisomal membrane	oxidative stress, hydrogen peroxide removal, hydrogen peroxide catabolic process, oxidation-reduction process	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell
APX6	At4g32320	cytosol, extracellular region	hydrogen peroxide catabolic process, oxidative stress, seed germination, seed maturation	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell
sAPX	At4g08390	chloroplast stroma, mitochondrion membrane	hydrogen peroxide catabolic process, oxidation-reduction process, cytokinin, oxidative stress	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell
tAPX	At1g77490	integral component of chloroplast thylakoid membrane	chloroplast-nucleus signaling pathway, cold acclimation, hydrogen peroxide catabolic process, hydrogen peroxide mediated signaling pathway, oxidation-reduction process, oxidative stress	root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell

Plant species and their APX isoforms are visible in figures representing the phylogenetic tree of APX proteins. Table 2 shows the cellular localization of the studied 45 plant species.

Table 2.

List of analyzed species, APX isoforms and their subcellular localization

	Species name	**NCBI GenBank ID**	**APX**	**Subcellular localization**
1	*Arabidopsis thaliana*	AEE28201.1	APX1	Cytoplasmic
2	*Arabidopsis thaliana*	Q05431.2	APX1	Cytoplasmic
3	*Beta vulgaris* subsp. *vulgaris*	XP_010691257.1	APX1	Cytoplasmic
4	*Brachypodium distachyon*	XP_003558178.1	APX1	Cytoplasmic
5	*Brassica rapa*	XP_009118405.1	APX1	Cytoplasmic
6	*Citrus maxima*	ACM17464.1	APX1	Cytoplasmic
7	*Cucumis sativus*	AGJ72850.1	APX1	Cytoplasmic
8	*Gossypium hirsutum*	ABR18607.1	APX1	Cytoplasmic
9	*Jatropha curcas*	ACV50426.1	APX1	Cytoplasmic
10	*Malus domestica*	ABP87792.1	APX1	Cytoplasmic
11	*Morus notabilis*	EXC33221.1	APX1	Cytoplasmic
12	*Nicotiana sylvestris*	XP_009784425.1	APX1	Cytoplasmic
13	*Nicotiana tabacum*	AAA86689.1	APX1	Cytoplasmic
14	*Nicotiana tomentosiformis*	XP_009597491.1	APX1	Cytoplasmic
15	*Oryza brachyantha*	XP_006649890.1	APX1	Cytoplasmic
16	*Oryza sativa Japonica*	ABF95353.1	APX1	Cytoplasmic
17	*Oryza sativa Japonica*	Q10N21.1	APX1	Cytoplasmic
18	*Populus euphratica*	XP_011008849.1	APX1	Cytoplasmic
19	*Prunus mume*	XP_008224940.1	APX1	Cytoplasmic
20	*Sesamum indicum*	XP_011089855.1	APX1	Cytoplasmic
21	*Setaria italica*	XP_004984819.1	APX1	Cytoplasmic
22	*Solanum lycopersicum*	AAZ77770.1	APX1	Cytoplasmic
23	*Solanum tuberosum*	NP_001275066.1	APX1	Cytoplasmic
24	*Spinacia oleracea*	BAA12890.1	APX1	Cytoplasmic
25	*Tarenaya hassleriana*	XP_010521780.1	APX1	Cytoplasmic
26	*Vitis vinifera*	NP_001267988.1	APX1	Cytoplasmic
27	*Zea mays*	NP_001152249.1	APX1	Cytoplasmic
28	*Aegilops tauschii*	EMT09178.1	APX2	Cytoplasmic
29	*Arabidopsis thaliana*	AEE74792.1	APX2	Cytoplasmic
30	*Arabidopsis thaliana*	Q1PER6.3	APX2	Cytoplasmic
31	*Beta vulgaris* subsp. *vulgaris*	XP_010696372.1	APX2	Cytoplasmic
32	*Brachypodium distachyon*	XP_003562395.1	APX2	Cytoplasmic
33	*Brassica rapa*	XP_009123280.2	APX2	Cytoplasmic
34	*Brassica rapa* subsp. *oleifera*	CCC55736.1	APX2	Cytoplasmic
35	*Camelina sativa*	XP_010486536.1	APX2	Cytoplasmic
36	*Citrus maxima*	ACM17463.1	APX2	Cytoplasmic
37	*Citrus sinensis*	XP_006480586.1	APX2	Cytoplasmic
38	*Fragaria vesca* subsp. *vesca*	XP_004302839.1	APX2	Cytoplasmic
39	*Glycine max*	AAB01221.1	APX2	Cytoplasmic
40	*Gossypium arboreum*	KHG05754.1	APX2	Cytoplasmic
41	*Malus domestica*	XP_008350397.1	APX2	Cytoplasmic

	Species name	**NCBI GenBank ID**	**APX**	**Subcellular localization**
42	*Momordica charantia*	AIE12238.1	APX2	Cytoplasmic
43	*Nelumbo nucifera*	XP_010253495.1	APX2	Cytoplasmic
44	*Oryza brachyantha*	XP_006658179.1	APX2	Cytoplasmic
45	*Oryza sativa Japonica*	Q9FE01.1	APX2	Cytoplasmic
46	*Populus euphratica*	XP_011048406.1	APX2	Cytoplasmic
47	*Prunus mume*	XP_008239139.1	APX2	Cytoplasmic
48	*Sesamum indicum*	XP_011094725.1	APX2	Cytoplasmic
49	*Setaria italica*	XP_004958804.1	APX2	Cytoplasmic
50	*Tarenaya hassleriana*	XP_010543364.1	APX2	Cytoplasmic
51	*Theobroma cacao*	EOY07733.1	APX2	Cytoplasmic
52	*Theobroma cacao*	XP_007016653.2	APX2	Cytoplasmic
53	*Vitis vinifera*	XP_010651099.1	APX2	Cytoplasmic
54	*Zea mays*	NP_001105500.2	APX2	Cytoplasmic
55	*Arabidopsis lyrata* subsp. *lyrata*	XP_020874660.1	APX3	Cytoplasmic
56	*Arabidopsis thaliana*	Q42564.1	APX3	Cytoplasmic
57	*Beta vulgaris* subsp. *vulgaris*	XP_010679905.1	APX3	Mitochondrial
58	*Brassica rapa*	XP_009138334.1	APX3	Cytoplasmic
59	*Citrus sinensis*	XP_006486751.1	APX3	Cytoplasmic
60	*Nicotiana tomentosiformis*	XP_009618135.1	APX3	Cytoplasmic
61	*Oryza sativa Japonica*	Q0JEQ2.1	APX3	Cytoplasmic
62	*Ricinus communis*	XP_002530823.1	APX3	Cytoplasmic
63	*Sesamum indicum*	XP_011088597.1	APX3	Cytoplasmic
64	*Solanum tuberosum*	XP_006359692.1	APX3	Cytoplasmic
65	*Vitis vinifera*	XP_002278281.1	APX3	Cytoplasmic
66	*Aegilops tauschii*	EMT10887.1	APX4	Cytoplasmic
67	*Aegilops tauschii* subsp. *tauschii*	XP_020163634.1	APX4	Cytoplasmic
68	*Arabidopsis thaliana*	P82281.2	APX4	Chloroplast
69	*Brachypodium distachyon*	XP_003574893.1	APX4	Cytoplasmic
70	*Hordeum vulgare* subsp. *vulgare*	BAB62533.1	APX4	Cytoplasmic
71	*Oryza brachyantha*	XP_006659666.1	APX4	Chloroplast
72	*Oryza sativa Japonica*	Q6ZJJ1.1	APX4	Chloroplast
73	*Oryza sativa Japonica*	XP_015650808.1	APX4	Chloroplast
74	*Setaria italica*	XP_004974146.1	APX4	Cytoplasmic
75	*Sorghum bicolor*	XP_002444620.1	APX4	Cytoplasmic
76	*Stipa purpurea*	AJF34885.1	APX4	Cytoplasmic
77	*Zea mays*	NP_001132505.1	APX4	Cytoplasmic
78	*Arabidopsis lyrata* subsp. *lyrata*	XP_002869048.1	APX5	Cytoplasmic
79	*Arabidopsis thaliana*	Q7XZP5.2	APX5	Cytoplasmic
80	*Arabidopsis thaliana*	NP_195321.1	APX5	Cytoplasmic
81	*Arabidopsis thaliana*	AAP72144.1	APX5	Cytoplasmic
82	*Brassica napus*	XP_013743324.1	APX5	Cytoplasmic
83	*Brassica rapa*	XP_009148286.2	APX5	Cytoplasmic
84	*Camelina sativa*	XP_010432206.1	APX5	Cytoplasmic
85	*Camelina sativa*	XP_010446847.1	APX5	Cytoplasmic

	Species name	**NCBI GenBank ID**	**APX**	**Subcellular localization**
86	*Capsella rubella*	XP_006285911.1	APX5	Nuclear
87	*Eutrema salsugineum*	XP_006412038.1	APX5	Nuclear
88	*Oryza sativa Japonica*	P0C0L0.1	APX5	Chloroplast
89	*Tarenaya hassleriana*	XP_010526807.1	APX5	Cytoplasmic
90	*Arabidopsis thaliana*	Q8GY91.1	APX6	Chloroplast
91	*Beta vulgaris* subsp. *vulgaris*	XP_010674956.1	APX6	Chloroplast
92	*Brachypodium distachyon*	XP_003578843.1	APX6	Mitochondrial
93	*Cucumis sativus*	XP_004149799.1	APX6	Chloroplast
94	*Fragaria vesca* subsp. *vesca*	XP_004290885.1	APX6	Chloroplast
95	*Jatropha curcas*	XP_012078304.1	APX6	Chloroplast
96	*Malus domestica*	XP_008365199.2	APX6	Chloroplast
97	*Nicotiana sylvestris*	XP_009784836.1	APX6	Chloroplast
98	*Nicotiana tomentosiformis*	XP_009629390.1	APX6	Chloroplast
99	*Oryza sativa Japonica*	P0C0L1.1	APX6	Mitochondrial
100	*Oryza sativa Japonica*	ABA96617.1	APX6	Mitochondrial
101	*Populus euphratica*	XP_010999402.1	APX6	Chloroplast
102	*Prunus mume*	XP_008219620.1	APX6	Chloroplast
103	*Sesamum indicum*	XP_011074839.1	APX6	Chloroplast
104	*Setaria italica*	XP_004977222.1	APX6	Mitochondrial
105	*Solanum lycopersicum*	NP_001234631.2	APX6	Chloroplast
106	*Vitis vinifera*	XP_003634424.1	APX6	Chloroplast
107	*Aegilops tauschii* subsp. *tauschii*	XP_020162413.1	sAPX	Chloroplast
108	*Arabidopsis thaliana*	Q42592.2	sAPX	Chloroplast
109	*Brachypodium distachyon*	XP_003579783.1	sAPX	Chloroplast
110	*Hordeum vulgare* subsp. *vulgare*	BAJ97403.1	sAPX	Chloroplast
111	*Oryza brachyantha*	XP_006652305.1	sAPX	Chloroplast
112	*Oryza sativa*	CAH67301.1	sAPX	Chloroplast
113	*Oryza sativa Indica Group*	EEC77311.1	sAPX	Chloroplast
114	*Oryza sativa Japonica*	Q7XJ02.1	sAPX	Chloroplast
115	*Oryza sativa Japonica*	XP_015635863.1	sAPX	Chloroplast
116	*Setaria italica*	XP_004975656.1	sAPX	Mitochondrial
117	*Sorghum bicolor*	XP_021319656.1	sAPX	Chloroplast
118	*Aegilops tauschii* subsp. *tauschii*	XP_020185648.1	tAPX	Chloroplast
119	*Arabidopsis thaliana*	Q42593.2	tAPX	Chloroplast
120	*Brachypodium distachyon*	XP_010235542.1	tAPX	Chloroplast
121	*Oryza brachyantha*	XP_006647364.1	tAPX	Chloroplast
122	*Oryza sativa Japonica*	Q69SV0.2	tAPX	Chloroplast
123	*Saccharum hybrid cultivar*	AGD80597.1	tAPX	Chloroplast
124	*Setaria italica*	XP_004952823.1	tAPX	Chloroplast
125	*Sorghum bicolor*	XP_021316117.1	tAPX	Chloroplast
126	*Triticum aestivum*	AAS80158.1	tAPX	Chloroplast
127	*Triticum urartu*	EMS46926.1	tAPX	Chloroplast

Phylogeny of APX isoforms out of 45 flowering plant species is represented by Figure 2.

The APX isoforms constitute a monophyletic group. The APX phylogenetic tree has split three branches with highly supported values (1 SH). Red band indicates the phylogenesis of APX1 and APX2 proteins, with highest number of species. These groups APX1 and APX2 sequences are mixed. The first lineage (0.677 SH-like support value) consists of species like Vitaceae, Pedaliaceae, Brassicaceae, Capparaceae, Malvaceae, Chenopodiaceae, Rutaceae, Rosaceae, Salicaceae families. Rutaceae family members has sister groups with Chenopodiaceae (APX2), Malvaceae (APX2), Capparaceae (APX2) and Brassicaceae (APX2) family species. The next lineage, Liliopsida class, consists of Poaceae family with APX1-2 groups, and they are sister groups (0.872 SH). Inside the Poaceae family there is a split between APX1 and APX2. The next split is the Malvales class with the highest confidence interval (0.780 SH). In close relation (0.8 SH) appears the Rosaceae with Moraceae and Cucurbitaceae family species. These groups are sister groups (0.826 SH) with Chenopodiaceae, Solanaceae, Brassicaceae, Capparaceae, Pedaliaceae family species. The second big branching are APX3, 4, 5 groups (1SH). The light green band shows the APX3 as a well-supported clade (0.895 SH) with three divergences: Chenopodiaceae, Brassicaceae and the close related Solanaceae, Vitaceae, Euphorbiaceae, Rutaceae, Pedaliaceae (0.895 SH) families. Another well supported clade is the APX4 marked with dark blue band, which comprise Poaceae family species and is separated in two sister groups with the highest divergence (1 SH). A well separated group is the APX5, colored with light green band, and consists the Capparaceae family and a highly separated Brassicaceae family species (0.998 SH). The last big branching is APX6, sAPX and tAPX groups (1 SH). APX6 is marked with light blue and is a well separated clade. The first clade is Liliopsida class (0.97 SH) with a well-supported clade, Poaceae family members (0.966 SH). The next branch is composed of Chenopodiaceae, Euphorbiaceae, Vitaceae, Rosaceae, Salicaceae, Pedaliaceae, Solanaceae, Cucurbitaceae families. The Vitaceae and Cucurbitaceae generates a sibling group and are consisted of one sister group, the Rosaceae family species. sAPX appears as a well-supported clade marked with lilac band. sAPX of *Oryza* genus are weakly supported. The yellow tAPX are well isolated group (1 SH), with two sister clades: *Oryza* genus and the Poaceae family species (0.375 SH). However, the low support of nodes on the phylogenetic tree shows that the relationship between the species is not clearly resolved yet. The high level of differentiation of APX groups is also confirmed by the high p-distance value expressed as a percentage value and appears as a high level of divergence between groups (Table 3).

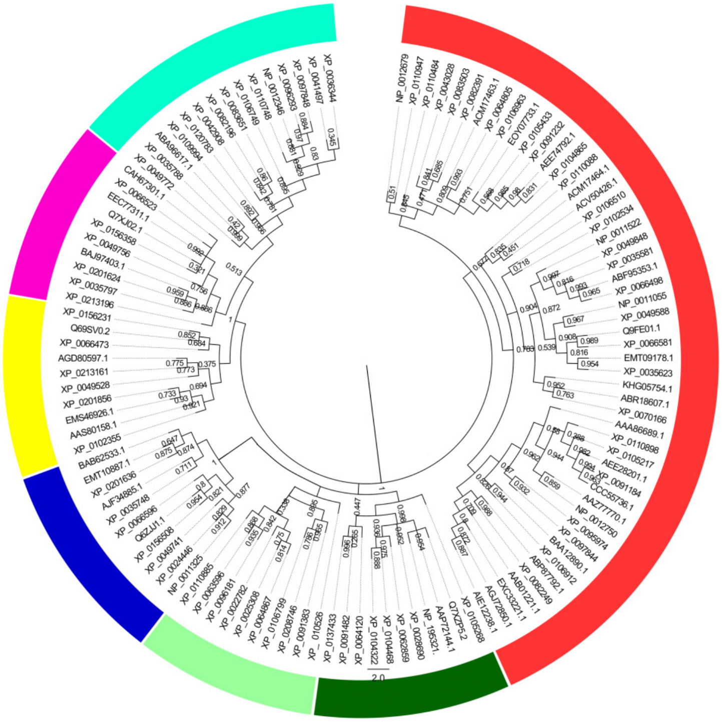

Figure 2. PhyML phylogenetic tree of 45 flowering plant species APX proteins; APX1 and APX2 are marked with red band, APX3 with light green band, APX4 with dark blue band, APX5 with light green band, APX6 with light blue band, sAPX with lilac band and tAPX with yellow band.

Table 3.

Average genetic distance between APX groups (%)

Groups name	APX1	APX2	APX3	APX4	APX5	APX6	sAPX
APX2	16.7						
APX3	33.1	33.2					
APX4	33.6	33.4	19.1				
APX5	38.7	38.3	26.8	31.8			
APX6	50.7	50.6	46.2	44.9	51.7		
sAPX	49.6	49.3	45.5	45.3	50.5	16.4	
tAPX	50.3	50.4	46.3	45.5	52.4	16.0	12.2

The genetic distance analyses within groups were also analyzed. The groups APX1-2-3 and APX6 revealed the higher genetic distance, exceeding the value of 1.1% (Table 4).

Table 4.

Average genetic distance within the APX groups (%)

Groups name	Genetic distance
APX1	1.59
APX2	1.54
APX3	1.19
APX4	0.66
APX5	0.90
APX6	1.26
sAPX	0.58
tAPX	0.55

Our cellular localization analysis indicated that APX1 and APX2 are localized in cytoplasm, APX3, APX4 and APX5 are localized in mitochondria, chloroplasts, nucleus and cytoplasm, APX6, sAPX and tAPX are mitochondrial and chloroplastic. The result of phylogeny reconstruction shows the relationship between the APX isoforms which may be influenced by the cellular localization also.

Conclusions

Ascorbate peroxidase is an essential enzyme in detoxifying the extremely harmful hydrogen peroxide, therefore in plant oxidative stress response. Molecular phylogeny analysis of several plant APX proteins has been presented in this study, in order to investigate the molecular manner of evolution of ascorbate peroxidase isoenzymes family in angiosperms. Evolutionary analysis of ascorbate peroxidase isoenzymes of different plant species, showed that APX is a monophyletic group, probably evolved from a single ancestor, where some isoformes are close related, some are not. Our phylogenetic analysis points close relationships between APX1 and APX2, between APX3, APX4 and APX5, and between APX6, sAPX and tAPX proteins in angiosperms. With an essential role in detoxifying processes of the cell, understanding molecular mechanisms of stress tolerance and phylogeny of APX, can serve to generate an efficient drug for prevention of several diseases in plants, animals and humans caused by severely harmful oxidative stress. Gathering information about molecular function of APX isoforms to scavenge ROS in different cellular compartments, could contribute to stress responsive gene engineering in order to improve crop tolerance against adverse environmental conditions.

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REFERENCES

Alscher, R. G., Donahue, H., Cramer, C. L. (1997) Reactive oxygen species and antioxidants: relationships in green cells, *Physiol. Plant.*, **100**:224–233

Apel, K., Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction, *Annu. Rev. Plant. Biol.,* **55**:373–399

Arvind, P., Prasad, M. N. V. (2003) Zinc alleviates cadmium-induced oxidative stress in *Ceratophyllum demersum* L: a free-floating freshwater macrophyte, *Plant. Physiol. Biochem.*, **41**:391-397

Begara-Morales, J. C., Sánchez-Calvo, B., Chaki, M., Valderrama, R., Mata-Pérez, C., López-Jaramillo, J., Padilla, M. N., Carreras, A., Corpas, F. J., Barroso, J. B. (2014) Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation, *J. Exp. Bot.*, **65**:527-538

Boo, Y. C., Yung J. (1999) Water deficit-induced oxidative stress and antioxidant defences in rice plants, *J. Plant Physiol.*, **155**:255-261

Campanella, J. J., Bitincka, L., Smalley, J. (2003) MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences, *BMC bioinformatics* 4:1-29

Chen, C., Dickman, M. B. (2005) Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii, Proc. Natl. Acad. Sci.*, **102**:3459-3464

Desikan, R., A-H-Mackerness, S. S., Hancock, J. T., Neill, S. J. (2001) Regulation of the Arabidopsis transcriptome by oxidative stress, *Plant Physiol.*, **127**:159–172

Elstner, E. F. (1991) Mechanisms of oxygen activation in different compartments of plant cells, In: *Active Oxygen/Oxidative Stress in Plant Metabolics*, Pelland, E.J., Steffen, K.L. (eds.), MD Rockville: American Society of Plant Physiologists, pp. 13-25

Foyer, C. H., Harbinson, J. (1994) Oxygen metabolism and the regulation of photosynthetic electron transport. In: *Causes* of *photo-oxidative stress and amelioration of defense systems in plants,* Foyer, C.H., Mullineaux, P.M. (eds.), CRC, Boca-Raton, pp. 1-42

Gill, S. S., Tuteja, N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, *Plant Physiol. Bioch.*, **48**:909-930

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0, *Syst. Bio.,* **59**:307-321

Halliwell, B. (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life, *Plant Physiol.*, **141**:312–322

Han, C., Liu, Q., Yang, Y. (2009) Short-term effects of experimental warming and enhanced ultraviolet-B radiation on photosynthesis and antioxidant defense of *Picea asperata* seedlings, *Plant Growth Regul.*, **58**:153–162

Hefny, M., Abdel-Kader, D. Z. (2009) Antioxidant-enzyme system as selection criteria for salt tolerance in forage sorghum genotypes (*Sorghum bicolor* L. Moench). In: *Salinity and Water Stress*, Ashraf, M., Ozturk, M., Athar, H.R. (eds.), editors. The Netherlands: Springer, pp. 25-36

Heyno, E., Mary, V., Schopfer, P., Krieger-Liszkay, A. (2011) Oxygen activation at the plasma membrane: relation between superoxide and hydroxyl radical production by isolated membranes, *Planta*, **234**:35–45

Jimenez, A., Hernandez, J. A., del Rio, L. A., Sevilla, F. (1997) Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Phys*., **114**:275–284

Khan, N. A., Samiullah, Singh S., Nazar, R. (2007) Activities of antioxidative enzymes, sulphur assimilation, photosynthetic activity and growth of wheat (*Triticum aestivum*) cultivars differing in yield potential under cadmium stress, *J. Agron. Crop. Sci.*, **193**:435-444

Klotz, L. O. (2002) Oxidant-induced signaling: effects of peroxynitrite and singlet oxygen, *Biol. Chem*., **383**:443–456

Kumar, S., Stecher, G., Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets, *Mol. Biol. Evol.*, **33**:1870-1874

Le, S. Q., Gascuel, O. (2008) An improved general amino acid replacement matrix, *Mol. Biol. Evol*., **25**:1307-1320

Lefort, V., Longueville, J. E., Gascuel, O. (2017) SMS: Smart Model Selection in PhyML, *Mol. Biol. Evol*., **34**:2422–2424

Madhusudhan, R., Ishikawa, T., Sawa, Y., Shigeoka, S., Shibata, H. (2003) Characterization of an ascorbate peroxidase in plastids of tobacco BY-2 cells. *Physiol. Plant*., **117**:550–557

Maheshwari, R., Dubey, R. S. (2009) Nickel-induced oxidative stress and the role of antioxidant defence in rice seedlings, *Plant. Growth. Regul*., **59**:37–49

Malan, C., Gregling, M. M., Gressel, J. (1990) Correlation between CuZn superoxide dismutase and glutathione reductase and environmental and xenobiotic stress tolerance in maize inbreeds, *Plant Sci*., **69**:157–166

McEntyre, J., Ostell, J. (2002) The NCBI handbook. Bethesda (MD): National Center for Biotechnology Information (US)

Mobin, M., Khan, N. A. (2007) Photosynthetic activity, pigment composition and antioxidative response of two mustard (*Brassica juncea*) cultivars differing in photosynthetic capacity subjected to cadmium stress, *J. Plant Physiol*., **164**:601-610

Nakano, Y., Asada, K. (1987) Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate depleted medium and reactivation by monodehydroascorbate radical, *Plant Cell Physiol*., **28**:131–140

Navrot, N., Rouhier, N., Gelhaye, E., Jaquot, J. P. (2007) Reactive oxygen species generation and antioxidant systems in plant mitochondria, *Physiol. Plant*., **129**: 185-195

Noctor, G., Foyer, C. H. (1998) Ascorbate and glutathione: keeping active oxygen under control, *Annu. Rev. Plant. Biol*., **49**:249–279

Orvar, B. L., Ellis, B. E. (1997) Des plants de tabac transgéniques exprimant un ARN antisens pour ascorbate peroxydase cytosolique montrent une susceptibilité accrue aux blessures d’ozone [Transgenic tobacco plants expressing an antisense RNA to cytosolic ascorbate peroxidase show increased susceptibility to ozone injury]. *L’usine J*., **11**:1297-1305

Sharma, P., Dubey, R. S. (2004) Ascorbate peroxidase from rice seedlings: properties of enzyme isoforms, effects of stresses and protective roles of osmolytes, *Plant Sci*., **167**:541–550

Sharma, P., Dubey, R. S. (2005a) Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: role of osmolytes as enzyme protectant, *Journal of Plant Phys*., **162**:854-864

Sharma, P., Dubey, R. S. (2005b) Drought induces oxidative stress and enhances the activities of antioxidant enzymes in growing rice seedlings, *Plant Growth Regul*., **46**:209–221

Sharma, P., Dubey, R. S. (2007) Involvement of oxidative stress and role of antioxidative defense system in growing rice seedlings exposed to toxic concentrations of aluminum, *Plant Cell Rep.*, **26**:2027–2038

Sharma, P., Jha, B., Dubey, R. S., Pessarakli, M. (2012) Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions, *J. Bot*., doi:10.1155/2012/217037

Simonovicova, M., Tamás, L., Huttová, J., Mistrík, I. (2004) Effect of aluminium on oxidative stress related enzymes activities in barley roots, *Biol. Plant*., **48**:261-266

Singh, S., Khan, N. A., Nazar, R., Anjum, N. A. (2008) Photosynthetic traits and activities of antioxidant enzymes in blackgram (*Vigna mungo* L. Hepper) under cadmium stress, *American J. Plant Phys*., **3**:25-32

Srivastava, A. K., Bhargava. P., Rai, L. C. (2005) Salinity and copper-induced oxidative damage and changes in antioxidative defense system of *Anabaena doliolum*, *World J. Microbiol. Biotechnol*., **22**:1291-1298

Székely, G., Ábrahám, E., Cséplő, Á., Rigó, G., Zsigmond, L., Csiszár, J., Ayaydin, F., Strizhov, N., Jásik, J., Schmelzer, E., Koncz, C., Szabados, L. (2008) Duplicated P5CS genes of *Arabidopsis* play distinct roles in stress regulation and developmental control of proline biosynthesis, *Plant J*., **53**:11-28

Tsugane, K., Kobayashi, K., Niwa, Y., Ohba, Y., Wada, K., Kobayashi, H. (1999) A recessive *Arabidopsis* mutant that grows photoautotrophically under salt stress shows enhanced active oxygen detoxification, *Plant Cell*, **11**:1195–1206

Wang, J., Zhang, H., Allen, R. D. (1999) Overexpression of an *Arabidopsis* peroxisomal ascorbate peroxidase gene in tobacco increases protection against oxidative stress, *Plant Cell Physiol*., **40**:725–732

Yan, J., Tsuichihara, N., Etoh, T., Iwai, S. (2007) Reactive oxygen species and nitric oxide are involved in ABA inhibition of stomatal opening, *Plant Cell Environ*., **30**:1320–1325

Yang, Y., Han, C., Liu, Q., Lin, B., Wang, J. (2008) Effect of drought and low light on growth and enzymatic antioxidant system of *Picea asperata* seedlings, *Acta Physiol. Plant*., **30**:433-440

Yu, C. S., Lin, C. J., Hwang, J. K. (2004) Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions, *Protein Science*, **13**:1402-1406

Yu, C. S., Chen, Y. C., Lu, C. H., Hwang, J. K. (2006) Prediction of protein subcellular localization, *Proteins: Structure, Function and Bioinformatics*, **64**:643-651

Zhengiang, L., Liu, D., Shenkui, L. (2007) Two rice cytosolic ascorbate peroxidases differentially improve salt tolerance in transgenic *Arabidopsis*, *Plant Cell Rep*., **26**:1909-1917

Zlatev, Z. S., Lidon, F. C., Ramalho, J. C., Yordanov, I. T. (2006) Comparison of resistance to drought of three bean cultivars, *Biol. Plant*., **50**:389-394.